Human Molecular Genetics, 2011, Vol. 20, No. 14 **2823–2833**
doi:10.1093/hmg/ddr193
Advance Access published on May 2, 2011

The liver receptor homolog-1 (LRH-1) is expressed in human islets and protects β-cells against stress-induced apoptosis

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Received February 25, 2011; Revised April 1, 2011; Accepted April 27, 2011

Liver receptor homolog (LRH-1) is an orphan nuclear receptor (NR5A2) that regulates cholesterol homeostasis and cell plasticity in endodermal-derived tissues. Estrogen increases LRH-1 expression conveying cell protection and proliferation. Independently, estrogen also protects isolated human islets against cytokine-induced apoptosis. Herein, we demonstrate that LRH-1 is expressed in islets, including β-cells, and that transcript levels are modulated by 17β-estradiol through the estrogen receptor (ER)α but not ERβ signaling pathway. Repression of LRH-1 by siRNA abrogated the protective effect conveyed by estrogen on rat islets against cytokines. Adenoviral-mediated overexpression of LRH-1 in human islets did not alter proliferation but conferred protection against cytokines and streptozotocin-induced apoptosis. Expression levels of the cell cycle genes *cyclin D1* and *cyclin E1* as well as the antiapoptotic gene *bcl-xl* were unaltered in LRH-1 expressing islets. In contrast, the steroidogenic enzymes CYP11A1 and CYP11B1 involved in glucocorticoid biosynthesis were both stimulated in transduced islets. In parallel, graded overexpression of LRH-1 dose-dependently impaired glucose-induced insulin secretion. Our results demonstrate the crucial role of the estrogen target gene *nr5a2* in protecting human islets against-stressed-induced apoptosis. We postulate that this effect is mediated through increased glucocorticoid production that blunts the pro-inflammatory response of islets.

INTRODUCTION

Diabetes mellitus is a major global health burden with 3.2 million deaths per year with six deaths attributable to diabetes or related conditions every minute (1). The disease is characterized by increased blood glucose resulting in secondary complications such as cardiovascular disease, kidney failure, retinopathy and neuropathy if not properly controlled (2). Two major etiologies are related to increased glycemia. The first is an autoimmune destruction of the pancreatic insulin-producing β-cells (Type 1 diabetes), while the second is associated with poor β-cell function and increased peripheral insulin resistance (Type 2 diabetes). Similar to Type 1, β-cell death is also observed in Type 2 diabetes (3).

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Novel treatment should therefore not only aim at increasing insulin secretion but also at reducing beta-cell death in the attempt to maintain a sufficient critical functional β-cell mass that can normalize blood glucose.

The liver receptor homolog-1 (LRH-1) or NR5A2 is a member of the Ftz-F1 sub-family of nuclear receptors that binds as a monomer to its target genes. This nuclear receptor plays a pivotal role in endodermal development by controlling expression of key transcription factors, such as Foxa2, HNF-4α and HNF-1α (4,5). More recently, LRH-1 was also identified as one of the five transcription factors (together with Oct4, Sox2, Klf4 and c-myc) capable of reprogramming murine somatic cells to induced pluripotent stem cells highlighting the fundamental function of this orphan nuclear receptor in regulating developmental gene networks (6). In adult mammals, the nuclear receptor is expressed in the liver (7), intestine (8) and ovaries (9). In these tissues, LRH-1 regulates expression of genes involved in cholesterol and bile acid metabolism as well as steroidogenesis and cell proliferation (10). LRH-1 is also expressed in the exocrine pancreas (11,12). Independent studies have failed to detect expression of LRH-1 in β-cells (11–13). However, a recent study suggests that LRH-1 is expressed in pancreatic islets (14). Consistent with this expression pattern, the pancreatic transcription factor PDX1 was shown to regulate LRH-1 expression during development (13). Although LRH-1 is considered an orphan receptor that possesses constitutive activity, small phospholipids such as phosphatidylethanolamine were shown to occupy the ligand pocket domain and to increase the LRH-1 activity (15). In addition, small bicyclic compounds were recently found to be efficient agonists of LRH-1 activity (16).

LRH-1 was shown to promote cell proliferation in pancreatic, gastric and hepatic cell lines (8,17). Suppression of LRH-1 by RNA interference provoked apoptosis in the hepatocellular carcinoma cell line BEL-7402 (18). LRH-1 was also found to mediate the 17β-estradiol/estrogen receptor α (ERα)-dependent proliferation/survival of MCF7 cells (19). LRH-1 also conveys a cell protective role by inhibiting inflammatory responses in the liver as well as in the gut (20,21). These effects appear to be mediated by the direct transcriptional inhibition of the interleukin (IL)-1β and IL-6 genes by LRH-1 with the concomitant activation of IL-1Ra as well as CYP11A1 and CYP11B1, two key genes involved in glucocorticoid biosynthesis (21,22). Interestingly, isolated human islets treated with 17β-estradiol were protected against proinflammatory cytokine and oxidative stress (H_2O_2)-induced cell death (23,24). Furthermore, ERα-deficient mice, independent of gender, were found to be more susceptible to islet β-cell apoptosis and prone to insulin-deficient diabetes subsequent to acute oxidative stress (25).

In view of the reduced β-cell mass in Type 2 diabetes and destruction of cells in Type 1 diabetes, the identification of genes conferring replicative and/or protective properties is a topic of high actuality for the development of cell regeneration therapy. The apparent capacity of LRH-1 to protect and stimulate cell proliferation combined with high expression levels in human islets provides a strong argument to investigate the potential implication of this nuclear receptor in β-cell function and survival. Thus, the present study aims at determining whether LRH-1 is expressed in the endocrine pancreas and to evaluate its potential impact on islet physiology. We show that mouse, rat and human islet β-cells express LRH-1 and that 17β-estradiol through the ERα transiently stimulates expression of the orphan nuclear receptor. Adenoviral-mediated overexpression of LRH-1 protects insulin-producing β-cells from apoptotic agents such as cytokines and streptozotocin, highlighting an unprecedented role of the orphan nuclear receptor in islet survival.

RESULTS

LRH-1 is expressed in pancreatic islet β-cells

To address the controversy whether LRH-1 is indeed expressed in pancreatic islet β-cells (11,12,14), we conducted quantitative RT-PCR (QT-PCR) and immunofluorescence studies on mouse, rat and human islets. We found that LRH-1 was expressed in mature rodent and human islets of Langerhans as well as in fluorescence-activated cell sorting (FACS)-purified β- and non-β-cells of these islets, albeit at lower levels than those detected in the control exocrine pancreas, liver and intestine samples (Fig. 1A). In contrast, the heart, kidney and muscle did not show detectable levels of LRH-1 mRNA. Co-immunofluorescence analysis performed using anti-LRH-1 and anti-insulin serum confirmed the presence of endogenous LRH-1 in β- (Fig. 1B) as well as non-β-cells. In mouse, rat and human dispersed islet cells, LRH-1 predominantly stained in sub-regions of the nuclei and cytoplasm. In contrast no staining was detected in both human and rat islets in the absence of the primary antibody, suggesting specificity of the anti-LRH-1 sera to the nuclear receptor (Supplementary Material, Fig. S1A). This premise was confirmed by western blot analysis performed on total protein extracts isolated from insulinoma INS-1E cells engineered to conditionally overexpress human LRH-1. Indeed, a unique specific band corresponding to the estimated molecular weight of the predominant form of LRH-1 (55 kd) was detected upon treatment of INS-1E with increasing concentrations of doxycycline (DOX) (Supplementary Material, Fig. S1B). Of note, similar to a recent study we were unable to detect endogenous LRH-1 by immunoblot analysis (26). Taken together, these results, clearly demonstrate that the nuclear receptor is expressed in rodent and human pancreatic islets and more importantly in the β-cell.

Estradiol stimulates LRH-1 expression via the ERα but not ERβ

In order to investigate the transcriptional regulation of LRH-1 in the endocrine pancreas, islets were exposed to 17β-estradiol, an estrogen derivative shown to transiently induce the expression of the orphan nuclear receptor and to promote cell proliferation in the breast cancer MCF7 cell line (19). LRH-1 transcript levels were transiently increased in MCF7 and in the insulin-secreting INS-1E cell lines (Supplementary Material, Fig. S2A and B), as well as in mouse, rat and human islets reaching maximal induction after 6 h of exposure to estradiol (approximately 3- and 2-fold in rat and human islets, respectively) (Fig. 2A). No

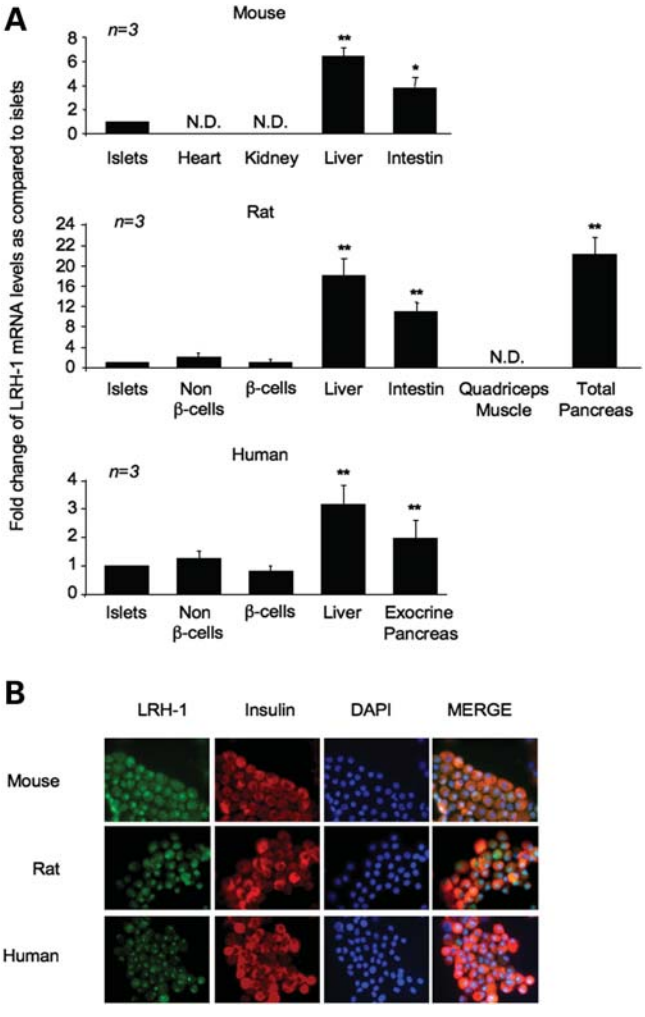

Figure 1. LRH-1 is expressed in pancreatic islet beta-cells (**A**) QT-PCR using RNA purified from isolated mouse, rat and human islets, FACS purified β- and non-β-cells as well as from various organs were performed to evaluate levels of LRH-1 transcripts. The average Ct value of LRH-1 for 50 ng of total RNA isolated from islets was estimated to be 26. Housekeeping genes' (Rps9 and cyclophilin) Ct values were approximately 18. Data are presented as fold change of LRH-1 mRNA levels as compared with islets. $^{**}P < 0.01$. N.D.; not detected. (**B**) Immunofluorescent detection of LRH-1 (green) and insulin (red) in dispersed adult mouse, rat and human islets. Nuclei were revealed using DAPI staining. Merged images confirm nuclear localization of LRH-1. 63× magnification.

differences were detected in estradiol-mediated stimulation of LRH-1 expression between genders in mouse islets. The induction was completely abrogated by the addition of the ER antagonist ICI 182 780 confirming a direct effect of the estrogen on LRH-1 expression. Of note, LRH-1 transcript levels were lower in ICI 182-treated islets as compared with control untreated islets, indicating that the ER signaling pathway is also involved in maintaining basal LRH-1 transcriptional levels (Fig. 2B). In order to further dissect the ER-mediated signaling pathway leading to increased LRH-1 expression, rat and human islets were incubated with the ERα agonist propylpyrazole triol (PPT) or the ERβ agonist diarylpropionitrile (DPN) (Fig. 2B). PPT induced an increase in LRH-1 mRNA levels similar to the condition with 17β-estradiol, whereas DPN had no significant effect on LRH-1 transcript levels. To confirm the functional role of ERα in regulating LRH-1 expression, islets isolated from ERαKO or ERβKO mice were incubated with 17β-estradiol (Fig. 2C). Consistent with the pharmacological data, LRH-1 transcript levels were not enhanced in ERαKO islets, whereas a 3-fold increase was detected in ERβKO as well as in control littermate islets treated with the hormone. Of note, although not statistically significant, a decrease in LRH-1 transcript levels was observed in islets isolated from ERα ablated transgenic animals suggesting that estrogens modulate basal levels of the orphan nuclear receptor. Our results thus show that the ERα-signaling pathway regulates LRH-1 expression in pancreatic islets.

17β-estradiol protects islets against cytokine-induced apoptosis partially through LRH-1 stimulation

Estradiol was shown to protect isolated human islets against cytokine- and oxidative stress-mediated cell death (23,24). In order to determine whether this effect was conveyed by LRH-1, siRNA was employed to suppress estrogen-induced LRH-1 expression in rat islets, which were then exposed to cytokines. LRH-1 transcript levels were inhibited by ∼70% in islets transfected with the LRH-1 siRNA (Fig. 3A). Induction of LRH-1 by estrogen was completely abrogated in these islets, whereas scrambled siRNA-expressing islets displayed a 2.5-fold increase in expression of LRH-1 (Fig. 3A). More importantly, LRH-1 suppression partially sensitized estrogen-treated islets to cytokine induced-apoptosis (Fig. 3B). Together, these results demonstrate that estradiol through ERα induces LRH-1 expression in islets resulting in increased protection against cytokine-evoked apoptosis.

Adenoviral-mediated overexpression of LRH-1 in islets does not alter proliferation

In order to gain further insight into the functional impact of LRH-1 on β-cell physiology, the transcription factor was conditionally overexpressed in islets using tetracycline inducible adenoviruses. Isolated human islets were co-infected with Ad-hLRH-1 and Ad-X Tet-On, and incubated for 48 h in the presence or absence of increasing concentration of the inducing tetracycline analog, doxycycline. QT-PCR established a dose-dependent stimulation of LRH-1 expression levels, reaching a 48-fold increase at 1 mg/ml doxycycline as compared with control untreated islets (Fig. 4A). A strong nuclear immunostaining was detected in 80% of human islet cells treated with doxycycline, while a diffused nuclear and cytoplasmic staining was observed in control islets (Fig. 4B). Similar results were obtained in rat islets as well as in the insulinoma INS-1E cell line (data not shown). As LRH-1 was previously shown to stimulate cell replication of pancreatic LTPA and hepatic FL83B cells (8), we next investigated the impact of LRH-1 overexpression on islet cell proliferation. Overexpression of LRH-1 did not alter cell proliferation in rat islets or human islets as compared with matched controls (Fig. 5).

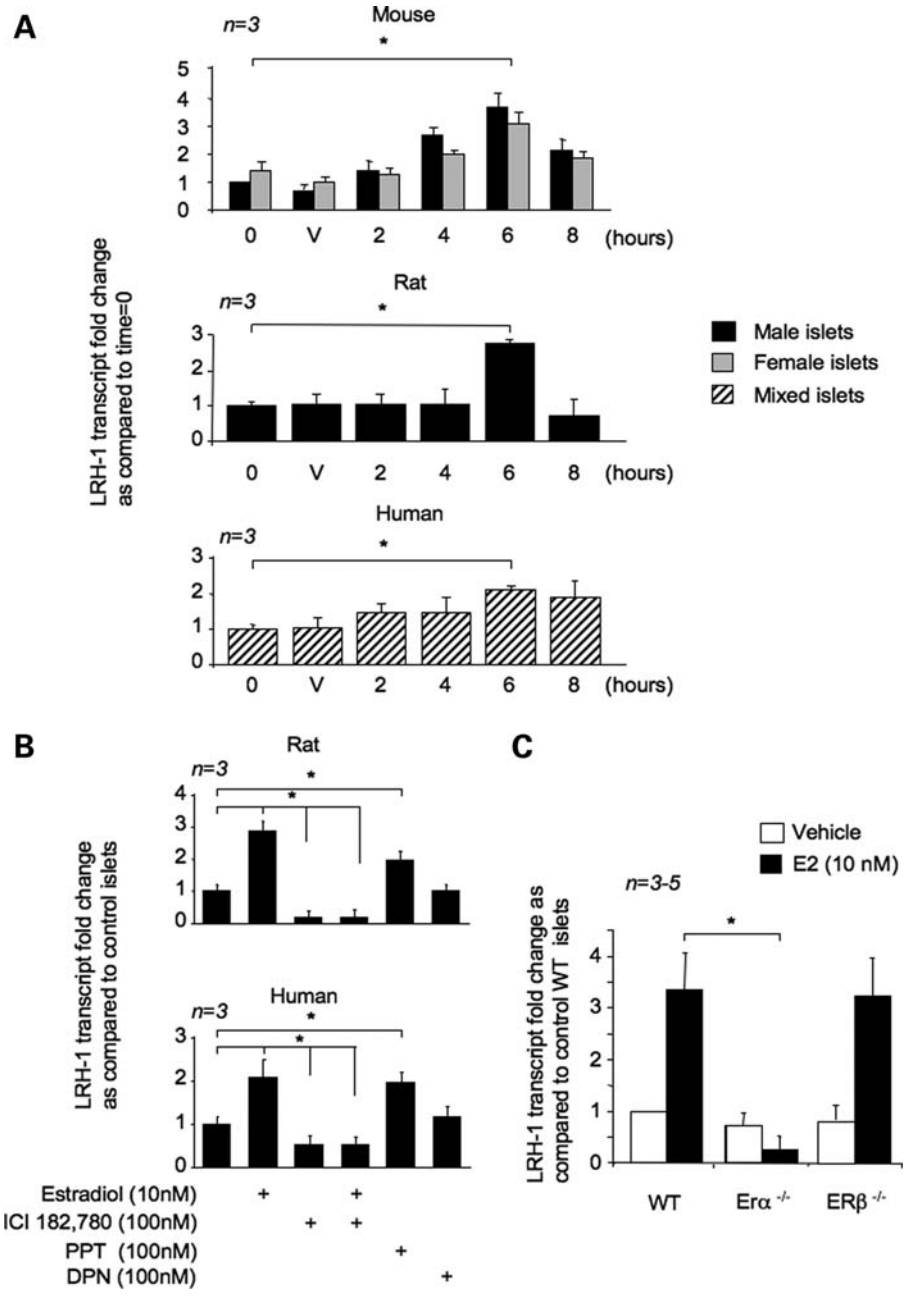

Figure 2. 17β-estradiol (E2) stimulates LRH-1 expression in islets via ERα (**A**) Isolated mouse, rat and human islets were incubated for 6 h with DMSO (vehicle) or for different times with 10 nM 17β-estradiol. (**B**) Isolated rat and human islets were incubated for 6 h with 10 nM 17β-estradiol, 100 nM of the ER antagonist ICI 182 780, the ERα agonist PPT or the ERβ agonist DPN. (**C**) Islets isolated from ERα knockout, ERβ knockout or wild type (WT) sibling mice were incubated for 6 h with DMSO (vehicle) or 10 nM 17β-estradiol. RNA was subsequently isolated and QT-PCR was performed to evaluate LRH-1 transcript levels. The average Ct value of LRH-1 for 50 ng of total RNA isolated from islets was estimated to be 26. Data are presented as fold change of LRH-1 mRNA levels as compared with untreated islets.

Rat and human islets overexpressing LRH-1 are protected against cytokine and streptozotocin-induced apoptosis

As a direct relationship between increased LRH-1 expression and islet survival in the presence of estrogen was observed (Fig. 2), we next evaluated the potential protective role of LRH-1 overexpression in rat and human islets exposed to either cytokines or streptozotocin. In humans, streptozotocin is used for reducing the size of islet carcinoma, most likely by increasing β-cell death (27). A 4-fold increase in TUNEL-positive β-cells was determined in control rat islets cultured in the presence of cytokines or streptozotocin. Remarkably, doxycycline-induced LRH-1 expression completely protected islets against apoptosis (Fig. 6A). Human islet β-cells exhibited a 10-fold increase in cytokine-mediated apoptosis, an effect that was dose dependently attenuated by increasing concentrations of doxycycline (Fig. 6B). LRH-1 overexpressing human islets were also refractory to apoptosis induced by

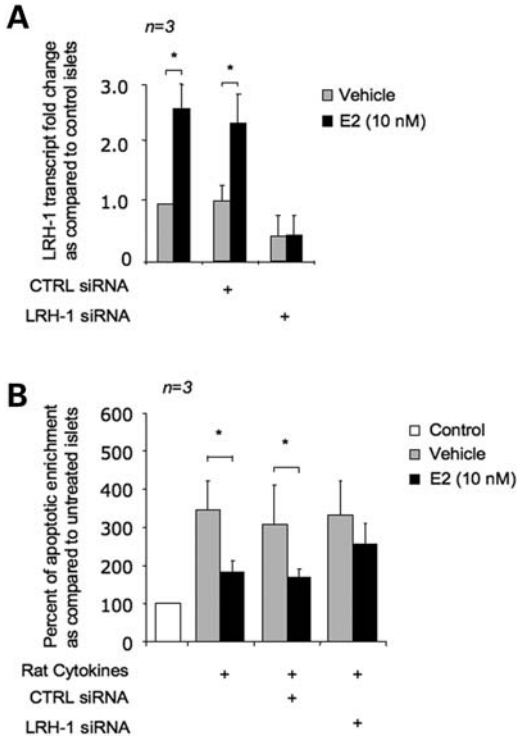

Figure 3. Targeted repression of LRH-1 abrogates the protective effect of estrogen on islets. (**A**) LRH-1 transcript levels were also evaluated by QT-PCR in rat islets transfected with either 50 nM of LRH-1 targeted siRNA or scrambled siRNA and incubated with (black bars) or without (grey bars) 10 nM 17β-estradiol. (**B**) siRNA-treated rat islets were challenged with a cocktail of cytokines (2 ng/ml rat IFN-γ, IL-1β and TNF-α) in the presence (black bars) or absence (grey bars) of 10 nM 17β-estradiol. Apoptosis was then measured using the Cell Death Detection ELISA kit. Data are presented as percentage of apoptotic enrichment compared with untreated islets. Each value represents the mean ± s.e. of three independent experiments performed in triplicates. $^{*}P < 0.05$.

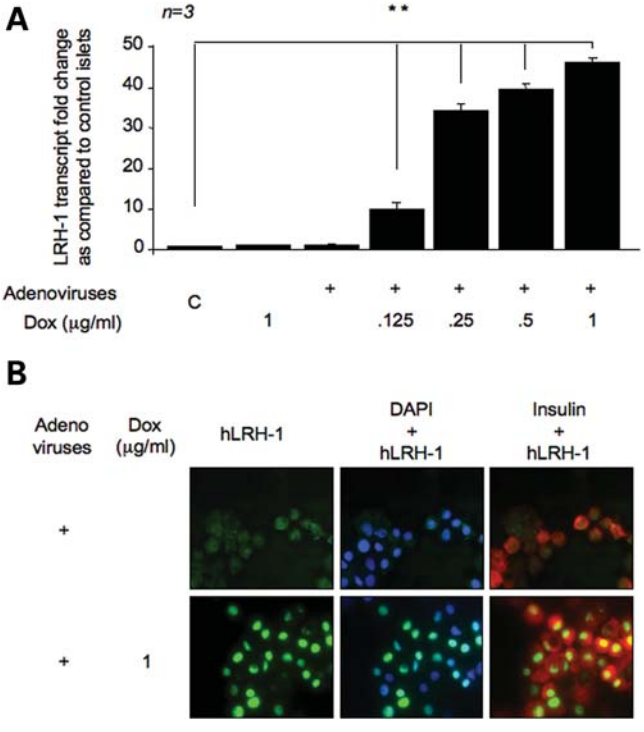

Figure 4. Doxycycline dose dependently increases LRH-1 transcript levels in human islets infected with Ad-hLRH-1. Human islets were transduced (or not) with Ad-hLRH-1 and Ad-X Tet-On and cultured in the presence of increasing concentrations of doxycycline for 48 h. (**A**) RNA was subsequently isolated and QT-PCR was performed to evaluate LRH-1 transcript levels. The average Ct value of LRH-1 for 50 ng of total RNA isolated from islets was estimated to be 26. Data are presented as fold change of LRH-1 mRNA levels as compared with untreated islets. Each value represents the mean ± s.e. of three independent experiments performed in duplicate. $^{**}P < 0.01$. (**B**) Immunofluorescence detection of LRH-1 (green) and insulin (red) as well as DAPI nuclei staining (blue) in dispersed human islet cells 48 h after infection with adenoviruses in the presence or absence of 1 μg/μl doxycycline. Merge images confirm nuclear localization of LRH-1. 63× magnification.

elevated doses of streptozotocin (Fig. 6C). As the TUNEL assay may be prone to false-positive results (28), we also measured apoptosis by monitoring cytoplasmic nucleosomes, using a cell death detection ELISA system and found similar results (Fig. 6D). Taken together these results clearly suggest that LRH-1 is involved in cell survival rather than cell proliferation in the endocrine pancreas. Furthermore, forced expression of LRH-1 recapitulates the effect observed with 17β-estradiol.

Transcript levels of the steroidogenic genes CYP11A1 and CYP11B1 are specifically increased in LRH-1 overexpressing islets

In an attempt to delineate potential mechanisms by which LRH-1 enhances β-cell viability, mRNA expression levels of various target genes were evaluated in islets overexpressing the orphan nuclear receptor. Consistent with the lack of proliferation, Cyclin D1 and E1 transcript levels were not significantly increased in LRH-1 transduced islets (Fig. 7A). Expression levels of the anti-apoptotic gene *bcl-xl* also remained constant (Fig. 7B). In contrast, mRNA levels for both CYP11A1 and CYP11B1 were increased by 14- and 4.5-folds, respectively, in LRH-1-transduced islets treated with the highest dose of DOX (Fig. 7C). These two genes encode cytochrome P450 enzymes, which are implicated in the generation of cortisol, the main glucocorticoid in humans (29).

Graded overexpression of LRH-1 attenuates glucose-induced insulin secretion in human islets

As glucocorticoids were shown to impair glucose-induced insulin secretion in human islets (30), we sought to determine whether overexpression of LRH-1 might blunt insulin secretion in infected islets. Consistent with this premise, graded overexpression of human LRH-1 resulted in a dose-dependent decrease in glucose-induced insulin secretion reaching a maximum inhibition of 60% as compared with non-treated islets at 1 μg/ml doxycycline (Fig. 8). Similar results were obtained when secreted insulin levels were normalized to DNA content rather than to total cellular insulin content (data not shown).

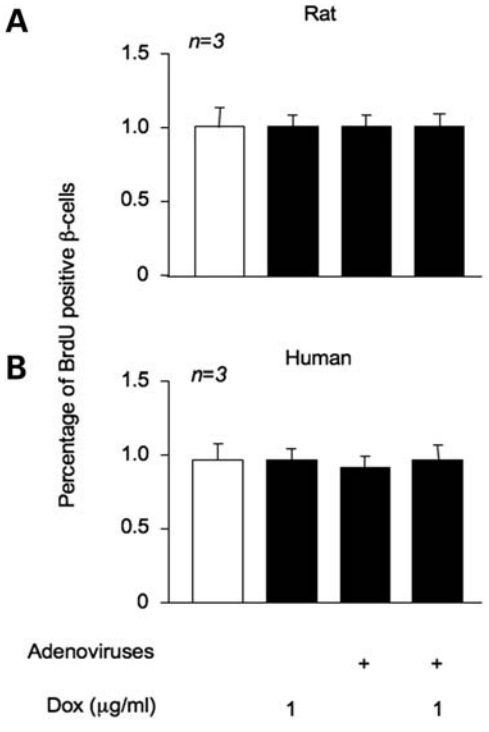

Figure 5. Overexpression of LRH-1 does not induce proliferation in islets. Proliferation was measured by BrdU incorporation in dispersed rat (**A**) or human (**B**) islets expressing LRH-1. Islets were transduced (or not) with Ad-hLRH-1 and Ad-X Tet-On, and cultured with or without 1 μg/ml of doxycycline for 48 h. Immunocytochemical detection of BrdU incorporation was performed 24 h after addition of BrdU (10 μM). Data are presented as percentage of BrdU positive β-cells as compared with the total number of β-cells. Each value represents the mean ± s.e. of three independent experiments performed in duplicates.

DISCUSSION

Identifying master genes controlling proliferation and survival pathways may uncover attractive targets for the development of novel therapies aimed at maintaining adequate β-cell mass in order to stabilize or even improve glucose metabolism in diabetic individuals. In this scope, the present study identifies the orphan nuclear receptor LRH-1 as a new islet transcription factor that conveys increased human islet β-cell survival in response to insults mimicking diabetes.

Consistent with a recent report (14), we detected the LRH-1 transcript in human and mouse islets and extended these findings to rat islets. Interestingly, Chuang *et al*. concluded that LRH-1 was exclusively expressed in α-cells as high levels were predominantly found in the mouse glucagonoma α-TC1 cell line while its expression was undetectable in the insulin-producing MIN6 cell line. However, we clearly demonstrate expression of LRH-1 in FACS-purified human and rat β-cells in addition to other islet cell types. Our findings in mouse islets are in sharp contrast with initial studies indicating that LRH-1 expression was confined to the exocrine compartment of the adult pancreas (11–13). These discrepancies may stem from different methodological approaches that allowed detection of low LRH-1 expression levels in mouse islets by QT-PCR (this study) but not by *in situ* hybridization (11,12). Although we cannot eliminate the possibility that islet

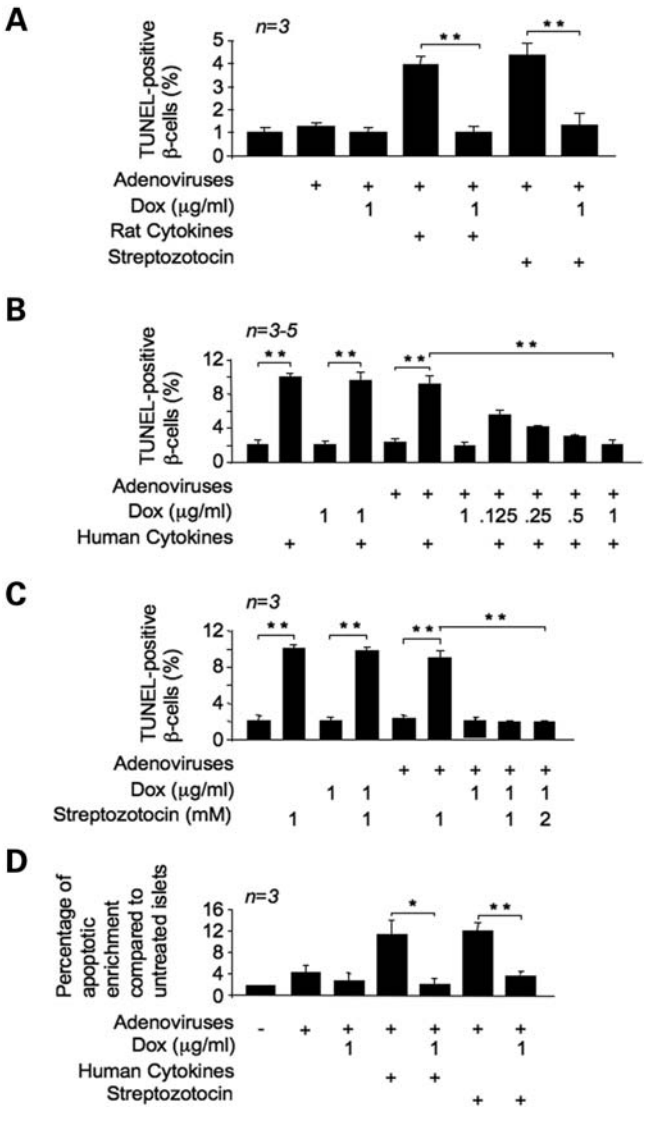

Figure 6. LRH-1 overexpression protects rat and human islets from either cytokine- or streptozotocin-induced apoptosis. Isolated rat (**A**) or human (**B**–**D**) islets were co-infected with Ad-hLRH-1 and Ad-X Tet-On, and cultured in the presence or absence of increasing concentrations of doxycycline as presented in the bar graph. Islets were subsequently treated for 24 h with rat (A) or human (B and D) 2 ng/ml IFN-γ, IL-1β and TNF-α to induce apoptosis. Alternatively, islets were treated with 1 (A, C and D) or 2 mM (C) of streptozotocin. (A–C) Cell death was measured by the TUNEL assay. Data are presented as percentage of TUNEL positive β-cells compared with the total amount of cells. More than 1000 cells were counted for each condition. (D) Alternatively, apoptosis was measured using the Cell Death Detection ELISA kit. Data are presented as percentage of apoptotic enrichment compared with untreated islets. Each value represents the mean ± s.e. of three independent experiments performed in triplicates. $^{*}P < 0.05$; $^{**}P < 0.01$.

preparations were contaminated with residual exocrine tissue that resulted in the erroneous detection of LRH-1 transcript, immunocytochemistry clearly established the presence of the LRH-1 protein in rodent and more importantly in human islets. These data were substantiated with a second anti-LRH-1 antibody reinforcing the concept that LRH-1 is expressed in the endocrine pancreas and more specifically in β-cells. The nuclear punctate pattern of LRH-1 detected in human islets

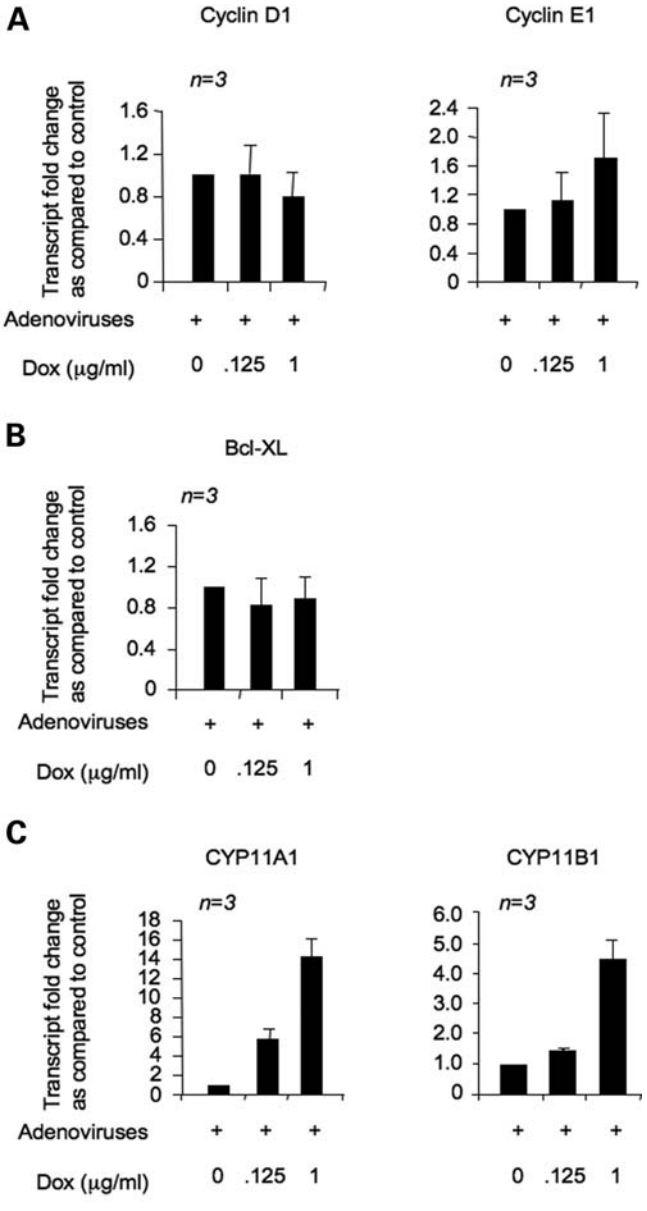

Figure 7. CYP11A1 and CYP11B1 expression levels are increased in human islets overexpressing LRH-1. Human islets were infected with Ad-hLRH-1 and Ad-X Tet-On and cultured in the presence of increasing concentrations of doxycycline for 48 h. Total RNA was then extracted and expression levels for (**A**) Cyclin D1 and Cyclin E1 as well as (**B**) Bcl-xL and (**C**) CYP11A1 and CYP11B1 were determined by QT-PCR. Each value represents the mean ± s.e. of three independent experiments. The average Ct values of Cyclin D1, Cyclin E1, Bcl-xL, CYP11A1 and CYP11B1 for 50 ng of total RNA isolated from islets were estimated to be 26, 31, 26, 34 and 30, respectively.

is reminiscent of that observed in rat granulosa cells and most likely stems from sumoylation of the receptor, a process regulated by cAMP signaling (31). Consistent with LRH-1 expression in islets, key pancreatic transcription factors such as Pdx1, FoxA2 and HNF-1α were shown to regulate expression of the orphan nuclear receptor during development and may thus also be involved in modulating expression in adult islets (13).

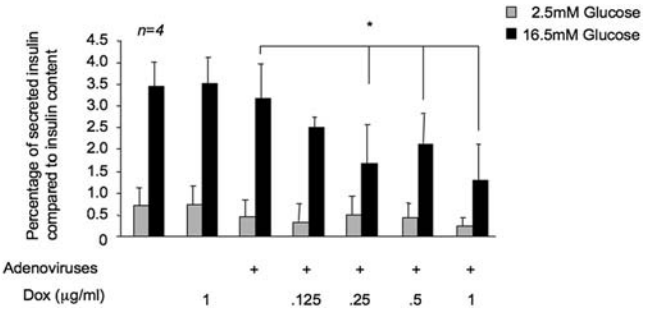

Figure 8. Graded LRH-1 overexpression impairs glucose-induced insulin secretion in human islets. Human islets were transduced (or not) with Ad-hLRH-1 and Ad-X Tet-On and cultured in the presence of increasing concentrations of doxycycline for 48 h. Insulin secretion was assessed in 30 min static incubations in response to 2.5 mM (grey bars) or 16.5 mM glucose (black bars). Insulin released in KRBH was quantified by EIA and expressed as a percentage of total cellular insulin content. Results are the mean ± s.e from three independent experiments performed in triplicates. $^{*}P < 0.05$.

We demonstrate that 17β-estradiol stimulates LRH-1 transcript levels in human and rodent islets. Pharmacological studies revealed the functional implication of the ERα but not ERβ in increased LRH-1 expression. Consistent with the latter, modulation of LRH-1 transcript levels by estrogens was completely abrogated in ERα-deficient mouse islets. More importantly, we identify LRH-1 as a downstream target of the estrogen signaling cascade that results in improved β-cell survival in the face of physiological stresses (23,24). Indeed, repression of LRH-1 sensitized estrogen-treated islets to cytokine-induced apoptosis. Our results initially appear at odds with a recent report demonstrating that ERα and β convey their protective effects on islets predominantly via rapid extranuclear mechanisms (24). Nonetheless, discrepancies may be resolved by the fact that Liu *et al.* performed an acute 5-h stress treatment, which exposed a rapid non-genomic ER action while we conducted a chronic 48-h stress treatment that may have revealed a more long-term ER-mediated transcriptional adaptation to noxious agents. Consistent with this premise, LRH-1 was recently shown to up-regulate ER expression in MCF7 cells suggesting a long-term positive regulatory feedback mechanism between ER and LRH-1 (26). Alternatively, LRH-1 expression levels may be potentially stimulated through the non-classical membrane-associated ERα rapid signaling pathway that potentiates gene expression through activation of extracellular signal-regulated kinases, mitogen-activated protein kinase and phosphoinositide 3-kinases (32).

The importance of LRH-1 in islet physiology was further highlighted using an inducible adenoviral vector in which expression of the orphan nuclear receptor was conditional on the addition of doxycycline to infected islets. Surprisingly, overexpression of LRH-1 did not induce cell proliferation in either rat or human islets. Independent studies have shown that LRH-1 expression is increased in gastric and breast cancers and that forced expression facilitates proliferation of pancreatic LTPA as well as hepatic FL83B cells in synergy with the β-catenin/TCF7L2 signaling pathway (8,17,19,33). Failure of causing islet cell proliferation may be a consequence of low expression levels of TCF7L2 and β-catenin in

mature islets, apparently mandatory in LRH-1-mediated cell replication (34,35). Consistent with the latter, expression levels of two LRH-1/β-catenin downstream target genes *cyclin D1* and *E1* (8) remained constant in LRH-1 expressing islets.

A major finding of the current work was the capacity of LRH-1 to protect islets against cytokine- or streptozotocin-induced apoptosis, two models of experimental diabetes. Surprisingly, screening of LRH-1-target genes revealed a robust induction of CYP11A1 and CYP11B1 expression levels, two transcripts encoding cytochrome P450 enzymes implicated in the biosynthesis of glucocorticoids. Glucocorticoids are potent immunosuppressive agents that blunt inflammation by inhibiting expression of cytokines with a concomitant increase in anti-inflammatory proteins (36). Historically, glucocorticoids were shown to be diabetogenic and to hamper islet function *in vivo* (37,38). These findings led to their exclusion from immuno-suppressive regimens used subsequent to islet transplantation (39). However, a recent study demonstrated that exogenous glucocorticoids have potent anti-inflammatory properties on human islets. Furthermore, although glucocorticoid-treated human islets exhibited a rapid reduction in glucose-induced insulin secretion observed within 24 h, these islets performed considerably better than control islets in long-term culture (30). Thus, it is tempting to speculate that LRH-1-mediated β-cell protection against cytokines could partially be conveyed by increased endogenous production of glucocorticoids resulting in decreased sensitivity of islets to the pro-inflammatory effects of cytokines with a concomitant impairment in glucose-induced insulin secretion. Further studies are currently underway to test this hypothesis. However, mild overexpression of LRH-1 induced by 0.125 μg/ml doxycycline considerably protected human islets against cytokines without impairing insulin secretion (Figs 4A, 6B and 8).

In summary, our study reveals LRH-1 expression in human, mouse and rat β-cells and establishes an unprecedented function for LRH-1 as a survival factor in these cells. Further delineating the molecular mechanism implicated in LRH-1-mediated resistance to apoptotic cues will facilitate development of novel therapies for the treatment of diabetes. The latter approach may prove to be more feasible than attempts at regeneration in view of recent findings suggesting that β-cell turnover is limited to the first three decades of human life (40,41).

MATERIALS AND METHODS

Mice and islet sources

Erα and β knockout (KO) mice have been described elsewhere (42). Animal experiments were performed in accordance with a Geneva Veterinary Cantonal Office- and a CABIMER-approved protocol. Pancreatic islets derived from 5-week-old male Wistar rats, C57BL/6J mice (Elevage Janvier, Le Genest-St-Isle, France) as well as Erα and βKO animals were isolated by collagenase digestion and hand picked as previously described (43). Human islets were obtained from The Cell Isolation and Transplantation Center (Department of Surgery, Geneva; Switzerland), the Department of Endocrinology (Metabolism, Metabolic Unit, Cisanello Hospital, Pisa); Italy and the Cell Therapy for Diabetes-CHRU de Lille, Lille; France. Islet preparations were washed, hand-picked and subsequently maintained in CMRL-1066 (Gibco) containing 5.6 mM glucose supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin. β- and non-β-cells were sorted by FACSVantage SE (Becton Dickenson) as described previously (44).

Cell line culture

Rat insulinoma INS-1E and MCF7 cells (45) were, respectively, cultured in RPMI-1640 and Dulbecco's modified Eagle's medium (Invitrogen, Basel, Switzerland) supplemented with 10% FCS and other additions as described previously (46).

Cell treatment

INS-1E cells, MCF7 cells or islets were cultured in phenol red-free medium containing 10% charcoal-stripped FCS for 48 h. The latter is nearly free of endogenous steroids to avoid skewing of the results. Islets were then cultured in the presence of 10 nM 17β-estradiol (Sigma-Aldrich), alone or in combination with 100 nM ICI 182 780 (ER antagonist; Tocris Bioscience), 100 nM PPT (ERα agonist; Tocris Bioscience) or DPN (ERβ agonist; Tocris Bioscience). For apoptosis studies, islets were exposed to a cocktail of cytokines (2 ng/ml IFN-γ, IL-1β and TNF-α) or streptozotocin (1 mM if not specified) for 48 h.

RNA interference

Fifty nanomolar of either ON-TARGETplus SMARTpool siRNA (Dharmacon, Inc., Chicago, USA) targeted to rat LRH-1 or ON-TARGETplus non-targeting pool was premixed with Lipofectamine (Invitrogen) and subsequently exposed to islets for 24 h. Fresh medium was then added and islets were exposed to cytokines for an additional 48 h.

LRH-1 adenoviral construction and transduction

The full-length human LRH-1 cDNA cloned into the expression vector pcDNA-T7tag was kindly provided by Lilly (Hamburg, Germany). Subsequently, LRH-1 cDNA was subcloned into the pTRE-Shuttle2 vector (CLONTECH Laboratories, Inc.). The inducible cassette was transferred into the Adeno-X viral DNA to generate the recombinant adenovirus Ad-hLRH-1. INS-1E cells were seeded at 3×10^5 cells/ml in 24-well plates, whereas 150 islets (rat or human) were used per experimental condition. Cells or islets were subsequently co-infected with Ad-hLRH-1 along with the adenoviral construct harboring the tetracycline transcriptional activator (Ad-X Tet-On) at a ratio of 2:1 (3.6×10^7 pfu/ml total viral particles). Cells were then transferred from 1.7 ml microfuge tubes to 24 well plates after either 90 min (islets) or 3 h (INS-1E) post-infection and cultured in fresh media supplemented with the indicated concentration of doxycycline.

Quantitative RT-PCR

Total RNA from various organs was extracted using the RNeasy Micro Kit (Qiagen) and 1–2 μg was converted into cDNA with the Superscript II Reverse transcriptase (Invitrogen). Primers were designed using the Primer Express software (Applera Europe, Rotkreuz, Switzerland) and sequences are provided in Supplementary Material, Table S1. Real-time PCR was performed using an ABI 7000 Sequence Detection System (Applera Europe), and PCR products were quantified fluorometrically using the FastStart Universal SYBR Green Master (Roche Diagnostics, Rotkreuz, Switzerland). Three distinct amplifications were performed in duplicate for each transcript, and mean values were normalized to the mean value of the reference mRNA cyclophilin and RPS9.

Immunohistochemistry

Control, treated and transduced islets were trypsinized to produce a single cell suspension and then cytospinned unto glass slides. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Endogenous and recombinant LRH-1 were visualized by immunohistochemistry using a mouse monoclonal antibody against human LRH-1 (Abcam, Cambridge, UK). Immunochemical detection of β-cells was performed as previously described (47). Nuclei were stained with diamidino-2-phenylindole (10 μg/ml; Sigma). Cover slips were mounted using DAKO fluorescent mounting medium and visualized using a Zeiss Axiophot I.

Proliferation and apoptosis assays

For proliferation, 10 μM BrdU was added to INS-1E cells for 3 h, and to rat or human islets for 24 h. Proliferation was estimated using an immunohistochemical assay kit as described by the manufacturer (BrdU labeling and detection Kit, Roche Diagnostics). Cell apoptosis induced either by cytokines or streptozotocin was measured using the TUNEL assay (In Situ Cell Death Detection Kit, Roche). Islet cells were also stained for insulin. Alternatively, the Cell Death Detection ELISA^{PLUS} (Roche) was used to quantify the degree of cytoplasmic histone-associated DNA fragments. Results of BrdU and TUNEL assays are expressed as a percentage of BrdU- or TUNEL-positive β-cells over the total amount of cells (nuclei staining by DAPI). Results of ELISA are presented as a percentage of apoptotic enrichment compared with untreated islets.

Glucose-stimulated insulin secretion

Twenty human islets/sample were preincubated with KRBH buffer (135 mM NaCl, 3.6 mM KCl, 2 mM NaHCO3, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 10 mM HEPES, pH 7.4 and 0.1% bovine serum albumin) containing 2.5 mM glucose for 30 min. Cells were then incubated for 30 min in KRBH buffer containing 2.5 mM glucose and an additional 30 min at 16.5 mM glucose. Insulin content, obtained by 10% acetic acid/ethanol (v/v) treatment of islets, and secreted insulin were quantified using the rat insulin enzyme immunoassay kit (Spi-Bio, Montigny le Bretonneux, France).

Statistical analysis

Results are expressed as mean ± SE. Where indicated, the statistical significance of the differences between groups was estimated by Student's *t*-test. $^{*}P$ and $^{**}P$ indicate statistical significance with 0.05 and 0.01, respectively.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of D. Brighouse, E. Husi and J. Cruz. We thank G. Kouri for adenoviral constructions, C. Zimmerman for providing transgenic animals, C. Kirkpatrick for kindly supplying RNA from FACS purified α-cells and Dr. Shomi Bhattacharya for his support as well as advice.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from the Swiss National Science Foundation (310030-119763 to B.R.G., 310000-116750/1 to C.B.W., 32003B-120376 to D.B.), the Juvenile Diabetes Research Foundation (9-2004-384 to E.C.I.T.), the Fundacion Progreso y Salud (to B.R.G.) and the Junta de Andalucia, Consejeria de Salud (PI-0727-2010 to B.R.G.).

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